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Short Communication

Extinction Coefficients of Chlorophyll *a* and *b* in *N,N*-Dimethylformamide and 80% Acetone[1]

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ABSTRACT

We found inconsistencies in the commonly used data for chlorophyll analysis in 80% acetone. Recently developed extinction coefficients for chlorophyll *b* in *N,N*-dimethylformamide (DMF) based on values from 80% acetone are low as a result of these inconsistencies. We determined extinction coefficients of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) in DMF for wavelengths of 618 to 665 nanometers. The simultaneous equations necessary for quantifying Chl *a*, Chl *b*, or total Chl in DMF in the absence of other chlorophyllous pigments are: Chl *a* = $12.70A_{664.5} - 2.79A_{647}$; Chl *b* = $20.70\ A_{647} - 4.62A_{664.5}$; total Chl = $17.90A_{647} + 8.08A_{664.5}$, where *A* = absorbance in 1.00 centimeter cuvettes and Chl = milligrams per liter.

***N,N*-Dimethylformamide is a very convenient solvent for Chl extraction since it is effective on intact plant parts and Chl is quite stable in DMF. There was no difference in the amount of Chl extracted when plant tissue was stored for 1 or 3 days at three temperatures, with or without solvent added.**

Analysis of Chl *a*, Chl *b*, or total Chl in plant tissue is commonly performed in 80% acetone after MacKinney (4). This method requires time consuming grinding and centrifugation steps. Moran and Porath (6) and Moran (5) developed an alternative method for Chl extraction from intact cotyledons using DMF[2]. Since then, other investigators have used this technique on intact leaves of *Gladiolus* (2) and soybeans (3).

The extinction coefficients necessary for the quantification of Chl *a*, Chl *b*, and total Chl in DMF were determined by Moran (5). The extinction coefficient for Chl *b* was determined using the coefficient for Chl *b* in 80% actone determined by MacKinney (4). This coefficient is not consistent with the results of Vernon (10) which suggest that if the equations developed by Moran (5) are used, then the calculations for Chl *b* content may be off by more than 10%. An error also would be introduced into the calculation of total Chl. One objective of this study was to determine which extinction coefficients were most consistent for Chl *a* and *b* in 80% acetone and DMF.

Both Chl *a* and Chl *b* are unstable to light and high temperatures and will degrade into their pheophytin counterparts (11). In DMF, Chl extracts were shown to be stable for up to 20 d when stored at 4°C in the dark (6). For DMF to be used as an extractant during field research, it is important to know additional nondestructive storage procedures for storing tissue samples until solvent extraction can be accomplished. The second objective of this study was to compare several different storage treatments of soybean leaf tissue and their effects on the quantity of Chl extracted.

MATERIALS AND METHODS

Measuring Extinction Coefficients. We obtained two 1 mg samples of Chl *a* (two different lots) and three 1 mg samples of Chl *b* (three different lots) from Sigma Chemical Co. We dissolved 1 mg samples of each Chl into approximately 2 ml of DMF. Using these as stock solutions, we transferred 0.10 ml aliquots into light-proof test tubes containing 3.00 to 20.00 ml of 80% (v/v) acetone, ether, or DMF. All solvents were spectroscopic grade. Normally, these transfers resulted in six different dilutions per solvent for each Chl sample, with absorbance values ranging from 0.02 to 1.7 depending on the chosen wavelength. We used a Beckman model 25 double beam spectrophotometer and 1.00 cm quartz cuvettes to measure absorbance values at wavelengths ranging from 618 to 665 nm. With one lot, we checked the absorbance values on a Beckman DU-2, and the two instruments agreed with 2%. The wavelength setting on the Beckman 25 was calibrated with a Holmium oxide filter (Beckman Instruments).

We used published extinction coefficients in ether (Sigma) (8) as a basis for calculating the concentrations of Chl *a* and Chl *b* in the original stock solutions. Using these concentrations, we calculated extinction coefficients of each Chl sample in 80% acetone and DMF for wavelengths ranging from 618 to 665 nm.

Plant Tissue Storage. We obtained 144 discs (d = 1.0 cm) from healthy soybean leaves (4th–5th trifoliate) that appeared similar in color, and established a 3 × 2 × 2 factorial experiment to compare three storage temperatures: room temperature, ice, and Dry Ice; two storage times: 1 and 3 d; and two storage phases: with and without 5 ml DMF added. We placed four discs into 36 light proof test tubes, and established the 12 treatment combinations, each treatment contained three replications. At the end of each storage time (5 ml DMF were added to those treatments without DMF), we shook each tube upright on a horizontal shaker for 24 h and read *A* at 664 nm in 1.00 cm cuvettes on the Beckman model 25 spectrophotometer.

RESULTS AND DISCUSSION

Extinction Coefficients. With the first lots of Chl *a* and *b*, we originally intended to use MacKinney's (4) extinction coefficients (ϵ) in 80% acetone to calculate the concentration of Chl *a* and *b* in the stock solutions. The concentration of Chl *a* in the stock solutions predicted from the values of MacKinney (4), Strain *et al.* (8), and Sigma Chemical Co. were very consistent (Table I).

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[2] Abbreviation: DMF, *N,N*-dimethylformamide.

Table I. *Calculated Concentration of Chl a in DMF Stock Solution Using Published Extinction Coefficients in 80% Acetone and Ether*

Solvent	Wavelength (λ)	Extinction Coefficient (ε)	Chl *a* Concn.[a]
	nm	$L \cdot g^{-1} \cdot cm^{-1}$	$g \cdot L^{-1}$
80% acetone (4)	645	16.75	0.571 ± 0.006
	663	82.04	0.580 ± 0.002
Ether (8)	661 (max)	96.59	0.566 ± 0.003
Ether (Sigma)	661 (max)	94.24	0.580 ± 0.003

[a] Average of four replications; ± values are SD.

Table II. *Calculated Concentration of Chl b in DMF Stock Solution Using Published Extinction Coefficients in 80% Acetone and Ether*

Solvent	Wavelength (λ)	Extinction Coefficient (ε)	Chl b Concn.[b]
	nm	$L \cdot g^{-1} \cdot cm^{-1}$	$g \cdot L^{-1}$
80% acetone[a] (4)	645	45.6	0.485 ± 0.006
	663	9.27	0.592 ± 0.008
80% acetone (10)	648 (max)	52.5	0.430 ± 0.002
	665	10.8	0.422 ± 0.002
Ether[a] (12)	643 (max)	54.34	0.478 ± 0.003
Ether (Sigma)	643 (max)	59.17	0.440 ± 0.002
Ether (8)	643 (max)	61.82	0.421 ± 0.002

[a] Extinction coefficients are from the same Chl b sample. [b] Mean of four replications; ± values are SD.

Table III. *Calculated Extinction Coefficients for Chl b in 80% Acetone*

80% Acetone	Wavelength (nm)							
	618	626	645	647 (max)	652	661	663	664.5
	$L \cdot g^{-1} \cdot cm^{-1}$							
ε[a]	10.24	11.64	50.00	51.21	43.38	16.39	12.41	10.20
CI[b]	0.17	0.18	0.67	0.47	0.50	0.30	0.22	0.22

[a] Chl *b* concentration determined in ether using ϵ_{max} = 59.17 (Sigma). [b] 99% confidence interval based on 10 replications.

However, we found a significant inconsistency in the concentrations predicted for Chl *b* using the coefficients at 663 and 645 nm (Table II). The extinction coefficients published by MacKinney (4) in 80% acetone and those published by Zscheile *et al.* (12) in either are from the same Chl *b* preparation. Both sets of extinction coefficients are significantly lower than other published values (8, 10; Sigma). In addition, the inconsistency between MacKinney's (4) coefficients at 663 and 645 nm suggests that either the Chl *b* samples we received from Sigma had a significant amount of Chl *a* or pheophytin *b* contamination, or that MacKinney's (4) samples were slightly impure.

The ratio of absorbance values, 643 nm (max)/520 nm for Chl *b* is very sensitive to slight degradation of Chl into pheophytin (11). Although Zscheile and Comar (11) reported ratios for Chl *b* near 18, Vernon (10) suggests ratios near 15. A range from 14 to 18 may therefore indicate only little degradation into pheophytin *b*. The ratios we found ranged from 14.8 to 16, indicating close agreement with Vernon's (10) pure Chl *b*. In addition, the ratios of the absorption at the 'blue' maximum (453 nm) to that at the 'red' (642 nm) one another indication of purity. All three lots of Chl *b* had ratios that ranged from 2.79 to 2.83, which was consistent with published values (8, 10). Therefore, we used the extinction coefficient of Sigma for Chl *b* in ether as a basis for calculating extinction coefficients in 80% acetone (Table III) and DMF (Table IV). If we had used the extinction coefficient from Strain *et al.* (8) as a standard, all calculated extinction coefficients (ε) for Chl *b* would be a factor of 1.04 higher (the ratio of ε, Strain/ε, Sigma). Calculated ε values for 80% acetone were significantly different from those published by MacKinney (4), and were in good agreement with Vernon (10). This suggests that the extinction coefficients for Chl *b* in DMF, calculated using MacKinney's coefficients (5), were also inconsistent.

Extinction coefficients of Chl *a* in DMF (Table IV) were similar to those in 80% acetone (4). The extinction coefficients for Chl *a* at 664 nm in DMF from other published data (5, 7) were normally within the 99% confidence interval of this study.

Because the Chl *a* and *b* spectra overlap, quantification of total Chl or the individual components is not possible unless two equations at two different wavelengths are established. A shortcut method for the calculation of total Chl is to choose the wavelength at which $\epsilon_{Chlb} = \epsilon_{Chla}$, as was done by Arnon (1) and Terry and Huston (9). At 652 nm, Arnon (1) found $\epsilon_a = \epsilon_b = 34.5$. This result was based on the lower extinction coefficients of MacKinney (4) and may be in error.

Our data in 80% acetone showed that the intersection occurred near 652.7 nm at which $\epsilon_a = \epsilon_b = 41$. This comparison brings up an important point concerning the reliance on ε values at wavelengths other than peak maxima. The intersection of Chl *a* and *b* extinction coefficients occurs on a very steep slope off the maximum, such that small deviations in wavelength settings will likely result in large errors. It is much safer to rely on the extinction coefficients at the wavelength maxima of each Chl component. These maxima are easily located by wavelength scans regardless of the spectrophotometer used.

We have solved the simultaneous equations necessary for

Table IV. *Calculated Extinction Coefficients for Chl b and Chl a in DMF*

Chl *b*	Wavelength (nm)							
	618	626	645	647 (max)	652	661	663	664.5
	$L \cdot g^{-1} \cdot cm^{-1}$							
ε[a]	9.83	10.81	49.05	50.81	43.47	17.75	13.62	11.17
CI[b]	0.16	0.12	0.47	0.52	0.48	0.35	0.31	0.28
n	11	11	16	16	11	8	16	16
Chl *a*	618 (max)	626	645	647	652	663	664.5 (max)	
ε[c]	16.02	14.34	15.72	18.47	33.81	81.95	82.80	
CI[b]	0.35	0.35	0.24	0.42	0.82	1.39	1.39	
n	9	9	18	9	9	18	18	

[a] Chl *b* concentration determined in ether using ϵ_{max} = 59.17 (Sigma). [b] 99% confidence interval based on *n* replications. [c] Chl *a* concentration determined in ether using ϵ_{max} = 59.17 (Sigma).

Table V. *Simultaneous Equations for Quantifying Chl in Plant Tissue from Measured Absorbance Values (A) Using 1.00 cm Cuvettes*

80% Acetone	DMF
Chl $b^a = 20.47A_{647}{}^b - 4.73A_{664.5}$	Chl $b = 20.70A_{647} - 4.62A_{664.5}$
Chl $a = 12.63A_{664.5} - 2.52A_{647}$	Chl $a = 12.70A_{664.5} - 2.79A_{647}$
Total Chl $= 17.95A_{647} + 7.90A_{664.5}$	Total Chl $= 17.90A_{647} + 8.08A_{664.5}$

[a] All Chl components in $mg \cdot L^{-1}$. [b] A_{647} = absorbance at 647 nm (maximum for Chl *b*); $A_{664.5}$= absorbance at 664.5 nm (maximum for Chl *a*)

Table VI. *Treatment Means of Absorbance Values Measured at 664 nm for Different Storage Treatments*

Temperature	Stored with Solvent		Stored without Solvent		Mean
	1 d	3 d	1 d	3 d	
Room Temp.	0.321[b]	0.288	0.297	0.324	0.308
Ice	0.289	0.303	0.310	0.312	0.304
Dry Ice	0.330	0.310	0.308	0.287	0.309
Mean[a]	0.313	0.300	0.305	0.308	

[a] P values for main effects: Temp = 0.88; Day = 0.57; Solvent = 0.96. [b] Three replications/treatment.

calculating Chl *a* and Chl *b* in 80% acetone and DMF (Table V.) MacKinney's (4) equations are based on absorbance at 663 nm and at 645 nm which are not the maxima for Chl *a* and Chl *b*. We chose to use the absorption maxima for Chl *a* and Chl *b* to minimize error between spectrophotometer settings. In DMF, the equation for Chl *a* was not significantly different from that presented by Moran (5) in the absence of PChl (a safe assumption in green mature tissue). However, due to the differences in extinction coefficients for Chl *b*, the equations for Chl *b* and the equation for total Chl were significantly different from those of Moran (5). The equations presented here only offter a correction for the extinction coefficients of Chl *b* in DMF. Moran (5) has performed a more thorough study including PChl which allows calculation of Chl contents in etiolated tissues. It should also be noted that Vernon (10) has established simultaneous equations in 80% acetone which require four absorbance readings at different wavelengths in order to account for possible conversion of either Chl *a* or Chl *b* to their pheophytin counterparts during sample preparation.

Tissue Storage. We found no significant differences (see P values) between the main effects of temperature of storage, length of storage, or storage with and without solvent on the absorbance values measured at 664 nm (Table VI). Any variability in the absorbance readings was probably due to differences between tissues discs and not differences between treatments. Provided that leaf tissue is stored in the dark, there is no need to add solvent for several days. Since Chl degrades at high temperature, it is advisable to store tissue at low temperature (5°C) until solvent extraction. Moran and Porath (6) also showed that DMF extracts stored in the dark at 4°C were stable for 20 d. These results demonstrate the convenience of DMF as a solvent for Chl extraction.

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